ATTACHMENT B

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(54) [Title of the Invention] IgE ANTIBODY PRODUCTION SUPPRESSOR AND ANTI-ALLERGIC AGENT

(57) [Abstract]
[Objects] A novel means for suppression of production of IgE antibody which plays an important role in the first stage of type I allergic reaction is provided whereby treatment and prevention of type I allergy are made easy.
[Constitution] An IgE antibody production suppressor and an anti-allergic agent in which cells of lactic acid bacteria are an effective ingredient.
[Claims]
[Claim 1] An IgE antibody production suppressor in which cells of lactic acid bacteria are effective ingredient.
[Claim 2] An anti-allergic agent in which cells of lactic acid bacteria are effective ingredient.
[Claim 3] The IgE antibody production suppressor according to claim 1, wherein the lactic acid bacteria are *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus kefir*, *Lactobacillus paracasei*, *Lactobacillus*

plantarum, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Streptococcus thermophylus*, *Lactococcus lactis*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, or *Enterococcus faecium*.

[Detailed Description of the Invention]

[0001]

[Industrial Field of Application]

The present invention relates to an IgE antibody production suppressor which is effective for prevention and treatment of allergy.

[0002]

[Prior Art]

Allergic diseases are classified into from type I to type IV according to their action mechanisms and the allergic disease, but the hay fever, atopic dermatitis, bronchial asthma, allergic rhinitis, allergic conjunctivitis and food allergy which causes distress to many people in Japan is an IgE antibody-dependent type I allergy.

[0003]

In an onset process of type I allergy, allergen-specific IgE antibody is firstly bonded to Fcε receptor on the surface of basophiles in blood and mast cells in tissues and then allergen is bonded to that IgE antibody, whereupon cross-link is formed between IgE antibodies. As a result of such a

cross-link, mast cells and basophiles are stimulated, whereupon chemical transmitters such as histamine, serotonin, leukotriene and heparin are liberated and these act to produce various allergic symptoms.

[0004]

Symptomatic treatment using anti-allergic agent a suppressing release of the above chemical transmitter, an anti-histaminic agent suppressing the effect of released chemical transmitter, a steroidal agent having an anti-inflammatory action, etc. have been carried out for type I allergy up to now but these conventional drugs have side effects to some extent, whereby there it is difficult to determine the method of their use.

[0005]

If production of the IgE antibody which plays an important role in the first stage of onset of type I allergy could be suppressed, that likely would be a fundamental prevention and treatment. However, effective means for suppression of production of IgE antibody has hardly been developed at all.

[0006]

[Problems that the Invention is to Solve]

Under such circumstances, an object of the present invention is to provide a novel means for suppression of production of IgE antibody, which plays an important role in

the first stage of type I allergic reaction, is provided, so that treatment and prevention of type I allergy are made easy.

[0007]

[Means for Solving the Problems]

The present invention which was successful in achieving the above object provides a suppressor for production of IgE antibody where cells of lactic acid bacteria are the effective ingredient, the present inventors being the first to find that cells of lactic acid bacteria suppress the production of IgE antibody.

[0008]

The present invention also provides a novel anti-allergic agent where cells of lactic acid bacteria are the effective ingredient, in which production of IgE antibody is suppressed whereby type I allergy is prevented or treated.

[0009]

A suppressive action of lactic acid bacteria for production of IgE antibody has been confirmed by an antibody production test using spleen cells of mice which were previously immunized against ovalbumin antigen which is a representative food allergen (refer to the Examples which will be mentioned later).

[0010]

Although there are differences to some extent in the lactic acid bacteria's suppression of production of IgE

antibody depending upon bacteria species and bacteria strains, such an action has been noted in all of *Lactobacillus* and *Lactococcus* the present inventors have investigated. Accordingly, there is no limitation upon lactic acid bacteria type at all in the present invention, and any lactic acid bacteria such as of the genus *Lactobacillus*, genus *Streptococcus*, genus *Lactococcus*, genus *Leuconostoc*, and genus *Enterococcus* may be used.

[0011]

Specific examples of preferred lactic acid bacteria for the present invention having particularly significant suppressive action for production of IgE antibody are *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus kefir*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Streptococcus thermophylus*, *Lactococcus lactis*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Leuconostoc lactis*, *Leuconostoc mescenteroides*, *Enterococcus faecalis* and *Enterococcus faecium*. Preferred ones among them are those where production amount of IgE is suppressed to not more than 30 ng/ml and, more preferably, not more than 10 ng/ml in the test for suppressive action for production of IgE antibody which will be mentioned

later.

[0012]

Lactic acid bacteria which are separated by a cell-collecting means such as centrifugal separation from the cultured product prepared by incubation according to any conventional method for incubation of lactic acid bacteria may be used as they are for the present invention. For the manufacture of the preparation, excipient, stabilizer, corrigent, etc. may be appropriately mixed followed by freeze-drying or dead cells may be prepared by heating and drying. It is also possible to manufacture a preparation by mixing with any other drug to the extent that the suppressive action of lactic acid bacteria for production of IgE antibody is not disturbed. Examples of dosage form are powder, tablets and drinks.

[0013]

The suppressive agent for production of IgE antibody and the anti-allergic agent according to the present invention are usually administered by oral route. An appropriate dose is about 10 to 1,000 mg on the basis of cell weight per day for an adult. Acute toxicity and side effects upon administration for a long period have not been noted.

[0014]

The suppressive agent for production of IgE antibody and the anti-allergic agent according to the present invention are

useful for prevention and treatment of allergic disease such as hay fever, atopic dermatitis, bronchial asthma, allergic rhinitis, allergic conjunctivitis and food allergy.

[0015]

[Examples]

Hereunder, the present invention will be illustrated by showing the result of tests of suppression of production of IgE antibody by various lactic acid bacteria. Incidentally, the lactic acid bacteria used for the test were those separated from human feces and digestive tract or dairy-related lactic acid bacteria, prepared by incubation of on an MRS medium by a conventional method, washing three times by suspending in aseptic water and by centrifugal separation, heating at 100°C for 30 minutes and freeze-drying.

[0016]

Test method:

Female BALB/c mice of five weeks age were immunized with aluminum hydroxide adjuvant of 1 mg (100 μl) to which 50 μg of ovalbumin was adsorbed, the spleen was excised therefrom after two weeks and a unicellular floating liquid was prepared. This was incubated together with ovalbumin (20 μg) and heat-killed cells of lactic acid bacteria (0.04 μg) using an RPMI 1640 medium (containing 100 U/ml of crystalline penicillin G potassium and 100 μg/ml of streptomycin sulfate) containing 10% fetal bovine serum, 6×10^5/200 μl/well (a 96-well

flat-bottom microplate; Nunc). The incubation was conducted under the condition of 5% carbon dioxide gas at 37°C. The supernatant liquid of the culture on the 14th day was collected and the amount of IgE antibody therein was measured by a sandwich ELISA method. Then, monoclonal anti-mouse IgE antibody (R35-92; trade name: Rm-E-01P; Pharmingen), which is a primary antibody, was dissolved in a sodium carbonate buffer to make 5 μg/ml and then 50 μl thereof was added to a 96-well microplate and adsorbed at 4°C for one night. After that, blocking was conducted, 50 μl of the supernatant liquid of culture was added and the mixture was incubated at 37°C for 90 minutes. Then 50 μl of biotin-labeled anti-mouse monoclonal IgE antibody (LO-ME-2) diluted 200-fold with a physiological saline buffered with phosphate containing 0.5% Triton X-100 was added thereto as the secondary antibody and incubation was conducted at 37°C for 90 minutes. Then 50 μl of peroxidase-labeled streptoavidin (Serotec) diluted 400-fold with a physiological saline buffered with phosphate containing 0.5% of Triton X-100 was incubated at 37°C for 90 minutes. To 100 ml of a buffer for colorization (citric acid-phosphoric acid buffer) were added 40 mg of o-phenylenediamine and 20 μl of 30% aqueous hydrogen peroxide to form a substrate solution, 100 μl of the resulting substrate solution was added to a well, an enzymatic reaction was conducted, and absorbance at 492 nm was measured. From a

standard curve prepared from a monoclonal mouse IgE standard solution, the amount of IgE antibody (ng/ml) in the supernatant liquid of culture was determined.

[0017]

Result of the test is shown in Table 1.

[Table 1]

Lactic Acid Bacteria	Produced Amount of IgE (ng/ml)
Lactobacillus acidophilus (ATCC 4356)	28.12
Lactobacillus acidophilus (ATCC 4357)	8.09
Lactobacillus acidophilus (ATCC 11975)	8.36
Lactobacillus acidophilus (JCM 1028)	21.96
Lactobacillus acidophilus (JCM 1229)	10.49
Lactobacillus brevis (ATCC 14869)	10.68
Lactobacillus buchneri (ATCC 4005)	13.94
Lactobacillus casei (ATCC 393)	10.13
Lactobacillus delbrueckii (ATCC 11842)	9.51
Lactobacillus fermentum (ATCC 14931)	9.51
Lactobacillus gasseri (DSM 20234)	65.95
Lactobacillus helveticus (ATCC 15009)	28.56
Lactobacillus johnsonii (JCM 2012)	37.22
Lactobacillus kefir (NRIC 1693)	8.22
Lactobacillus paracasei (NCDO 151)	9.37
Lactobacillus plantarum (ATCC 14917)	12.93
Lactobacillus rhamnosus (ATCC 7469)	25.11
Lactobacillus salivarius (ATCC 11741)	8.00
Streptococcus thermophilus (ATCC 19258)	3.92
Streptococcus thermophilus (YIT 2001, FERM P-11891)	8.98
Streptococcus thermophilus (ATCC 14485)	8.68
Streptococcus thermophilus (YIT 2021)	2.00
Streptococcus thermophilus (NCDO 821)	4.53
Streptococcus thermophilus (ATCC 19287)	24.63
Lactococcus lactis (ATCC 19257)	28.78
Lactococcus plantarum (ATCC 43199)	5.76
Lactococcus raffinolactis (ATCC 43920)	5.45
Leuconostoc lactise (ATCC 119256)	4.30
Leuconostoc mescenteroides (ATCC 19254)	8.08
Enterococcus faecalis (ATCC 19433)	2.46
Enterococcus faecium (ATCC 19434)	18.11
Control (no lactic acid bacteria added)	100.81

[0018]

The same suppressive action for production of IgE

antibody as above was also confirmed for protein antibody other than ovalbumin such as pollen and tick antigen.

[0019]

[Advantages of the Invention]

The suppressive agent for suppressing production of IgE antigen and the anti-allergic agent according to the present invention comprise lactic acid bacteria which constitute human intestinal bacterial flora or lactic acid bacteria which have been utilized for a long time for the manufacture of dairy products. Therefore, they are safe even when orally ingested for a long period continuously and, further, they should exhibit synergistically the useful actions which lactic acid bacteria have been known to have such as the effect of calming intestinal disorder, anti-tumor action, anti-mutation action, immunostimulation action, hypotensive action, anti-ulcer action and hypocholesteremic action. Accordingly, when they are used, prevention and treatment of various kinds of allergic diseases become significantly easier.

ATTACHMENT C

Original Paper

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Effects of Probiotics on Allergic Rhinitis Induced by Japanese Cedar Pollen: Randomized Double-Blind, Placebo-Controlled Clinical Trial

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Key Words

Japanese cedar pollen · Allergic rhinitis · Probiotic · *Lactobacillus casei* · Fermented milk

Abstract

Background: *Lactobacillus casei* strain Shirota (LcS) has been found to exert antiallergic effects in animal experiments, but there is little information about its clinical effects in human patients with allergy. ***Methods:*** We performed a randomized double-blind, placebo-controlled study to investigate the effects of LcS in patients with allergic rhinitis triggered by Japanese cedar pollen (JCP). Participants were asked to drink fermented milk containing LcS (LcS group) or placebo (control group) for 8 weeks. Clinical symptoms and immunological parameters were compared between the two groups. ***Results:*** Symptom-medication scores (SMS) worsened in accordance with the increase in the amount of scattered JCP. In terms of the nasal and ocular SMS, there was no significant difference between the LcS group and the placebo group during the ingestion period. In the subgroup of patients with moderate-to-severe nasal symptom scores before starting the ingestion of test samples, supplementation with LcS tended to reduce nasal SMS. ***Conclusion:*** These results indicate that fermented milk containing LcS does not prevent allergic symptoms in patients sensitive to JCP, but may delay the occurrence of allergic symptoms in patients with moderate-to-severe nasal symptom scores.

Introduction

Recently, patients with allergic rhinitis have been increasing in Japan, and the incidence of allergic rhinitis caused by Japanese cedar pollen (JCP) is estimated to be in the range of 10–15% among Japanese people [1]. This tendency could be a serious problem from the standpoint of socioeconomic aspects, because the allergic symptoms are unpleasant for patients and often cause various disturbances in their ordinary life during the particular season. Moreover, the medical bill for treating allergic symptoms is not negligible.

The general treatment of allergic rhinitis is administration of antihistamines, laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy. These modalities are considered efficient but have some problems. Antihistamines sometimes cause side effects

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Table 1. Characteristics of the participants in the LcS group and the placebo group

Characteristics	LcS group	Placebo group	Significance
Number of patients (male:female)	55 (22:33)	54 (21:33)	NS
Age, years	39.3 ± 8.0	39.5 ± 10.9	NS
Total IgE, IU/ml	198.9 ± 273.8	160.0 ± 247.0	NS
Anti-JCP IgE, IU/ml	15.6 ± 21.5	14.0 ± 15.7	NS
Nasal SMS	1.33 ± 0.72	1.42 ± 0.88	NS
Ocular SMS	0.94 ± 1.01	0.95 ± 0.97	NS
Swelling of nasal mucosa	1.24 ± 1.00	1.20 ± 0.86	NS
Color of nasal mucosa	1.45 ± 0.90	1.52 ± 0.91	NS
Amount of mucus	0.89 ± 0.76	0.89 ± 0.66	NS
Nature of mucus	1.45 ± 1.32	1.50 ± 1.31	NS

NS = Not significant; SMS = symptom-medication score.

such as sleepiness, thirst or gastrointestinal disturbance, and their dose and timing of administration should be strictly controlled. Laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy cause a great burden to the patient, such as hospitalization, or sometimes a long ambulatory treatment period. Under these circumstances, food products to prevent or improve allergic symptoms that are easily available in ordinary life are required. Thus, yogurt, tea and herbs have been shown to potentially relieve the allergic symptoms [2–5].

Supplementation with milk fermented with *Lactobacillus paracasei* 33, *L. acidophilus* L-92 or *Bifidobacterium longum* BB536 has been shown to suppress the subjective symptoms and may modulate immunological parameters in allergic rhinitis patients [6–9]. These findings support the opinion that stabilization of the intestinal microflora by administration of probiotics may prevent the development of allergic rhinitis.

L. casei strain Shirota (LcS) suppresses the IgE production of splenocytes by enhanced interleukin 12 secretion by macrophages in vitro [10], and its administration prevents the elevation of the IgE level and induction of anaphylactic symptoms after sensitization with ovalbumin in animal models [11, 12]. Therefore, it is worthwhile examining whether LcS could improve allergic symptoms in humans. We evaluated the effect of fermented milk containing LcS in patients with allergic rhinitis to JCP in a randomized double-blind, placebo-controlled study. This report shows that ingestion of fermented milk containing LcS did not prevent clinical symptoms or abnormal immunological parameters in patients allergic to JCP, but may delay the occurrence of subjective symptoms in patients with moderate-to-severe nasal symptom scores.

Materials and Methods

Subjects

To carry out this study, we recruited participants enrolled for human studies in the nonirradiated subject bank of Soiken Inc. (Osaka, Japan), which is an organization for evaluating the functions of foods or food-derived materials in humans. We explained the aim and protocol of this study, asked if they were willing to participate and screened for subjects having specific IgE for JCP by a scratch test using allergen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) and a radioallergosorbent test. Exclusion criteria were as follows: use of antihistamines or antiallergic medication at the time of the screening test; any recent history of acute rhinitis, sinusitis, nasal polyp, hypertrophic rhinitis, septal deformity or asthma; severe disorder of the liver, kidney, heart, respiratory organs, endocrine glands or metabolism; treatment with hyposensitization therapy; frequently drinking dairy products containing lactic acid bacteria, and cow's milk allergy. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee, which is independent of Soiken Inc. Written informed consent was obtained from all the participants.

Study Design

The study was performed in a randomized double-blind, placebo-controlled manner. Experiments were performed from January 22 to April 15, 2005. Randomization was performed by doctors, who were not involved in this study design. All of the enrolled subjects were randomly assigned to the LcS group or the placebo group according to computer-generated permuted-block randomization. There was no obvious difference in the two groups (table 1). The LcS group drank fermented milk containing LcS (4×10^{10} CFU/80 ml) and the placebo group drank unfermented milk. The composition of fermented milk containing LcS and placebo was the same except that the placebo did not contain LcS, and the lactic acid level of placebo was adjusted to the level of fermented milk containing LcS. Before delivery to the participants, we confirmed that the fermented milk contained more than 5×10^8 CFU/ml of LcS, and both fermented milk and placebo had no contamination with other bacteria.

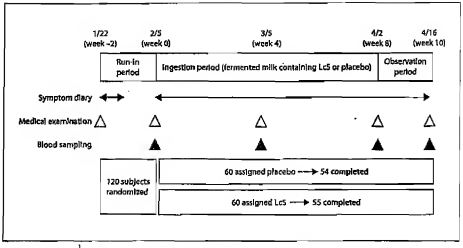

Fig. 1. Study protocol. The clinical trial was carried out from January 22 to April 15, 2005. Participants were asked to drink fermented milk containing LcS or placebo for 8 weeks, and to record their symptoms in a diary every day. Medical examination was conducted 5 times, and blood samples were taken 4 times during the study.

To assure the viability of LcS, we prepared fermented milk every week during the study period. Furthermore, we checked the acidity and sugar level in LcS-containing fermented milk and confirmed that these values were within standardized levels. We did not check the intestinal microflora in the volunteers in this study. However, it has already been verified that LcS can be detected in feces after supplementation of LcS-containing fermented milk [13–15]. We asked all the participants not to change their ordinary lifestyle during the study. The participants drank 80 ml of placebo or fermented milk containing LcS daily for 8 weeks. The schedule of the study is shown in figure 1. Participants were asked to record their nasal and ocular symptoms and medication in a diary during the study period. Moreover, they underwent medical examination by an otolaryngologist 5 times during the study.

Evaluation of Symptoms and Medical Examination of Subjects

The scores of nasal and ocular symptoms have been defined by the Japanese Society of Allergology [1, 16]. Briefly, sneezing, runny nose, stuffy nose, itchy eyes and watery eyes were each scored from 0 to 4 according to the severity of symptoms by the participants, and the medication score was estimated based on the efficacy of medicines (table 2). The medication score, which was described in the guidelines of the Japanese Society of Allergology, is determined by medication usage (table 2). The clinical condition of the nasal cavity (swelling and color of nasal mucosa, amount and nature of mucus) was scored from 0 to 3 for each feature according to the severity by the otolaryngologist (table 3). The symptom-medication score (SMS) was calculated by summing the symptom score and the medication score.

Table 2. Symptom score and medication score for evaluating the subjective symptoms

a Symptom score

Score	Sneezing	Runny nose	Stuffy nose	Itchy eyes	Watery eyes
0	0	0	none	none	none
1	1–5	1–5	mild	mild	mild
2	6–10	6–10	moderate	moderate	moderate
3	11–20	11–20	severe	severe	severe
4	>21	>21	violent	violent	violent

b Medication score

Score	Medicine
1	oral antihistamine, oral histamine release inhibitor, nose or eye drops (without steroids)
2	local administration of steroids
3	oral antihistamine plus local administration of steroids

Sneezing = Average number of sneezing attacks in a day; runny nose = average number of times patient blew nose in a day.

Table 3. Standard for doctors of otolaryngology to assess the nasal cavity

Score	Swelling of mucosa	Color of mucosa	Amount of mucus	Nature of mucus
0	none	normal	none	none
1	middle concha observable	light red	adhesion level	purulent
2	between 1 and 3	red	between 1 and 3	viscous
3	middle concha unobservable	bluish	filled	aqueous

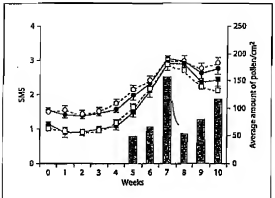

Fig. 2. Nasal and ocular SMS throughout the study period. Nasal SMS in the LcS group (●) and placebo group (○) and ocular SMS in the LcS group (■) and placebo group (□) are shown as means ± SE. Filled columns show the average amount of pollen (JCP and hinoki pollen) scattered in the area where the studies were carried out.

Blood Examination

Blood samples were collected 4 times during the study, and anti-JCP IgE, eosinophil number, eosinophil cationic protein (ECP) and the balance of Th1 cells to Th2 cells (Th1/Th2 ratio) were determined. The anti-JCP IgE level was evaluated by the radioallergosorbent test. The ECP level was measured by radioimmunoassay (Unicap system; Roche Diagnostics KK, Sweden). Peripheral blood mononuclear cells were stimulated with phorbol myristate acetate plus ionomycin for 4 h in the presence of brefeldin A, and stained with anti-CD4 antibody. After cells had been fixed and permeabilized, the accumulated γ-interferon and interleukin 4 in CD4+ T cells were stained and measured by a flow cytometer. The ratio of γ-interferon+ CD4+ T cells to interleukin-4+ CD4+ T cells was expressed as Th1/Th2 balance.

Statistical Analysis

SMS were averaged each week. Differences in SMS and description of the nasal mucosa between the groups were evaluated by the Mann-Whitney U test. Differences in immunological parameters were assessed by the unpaired Student's t test. Data were analyzed using SPSS software (version 11.5, SPSS Inc., Chicago, Ill., USA).

Results

Scattering of JCP

The Osaka Prefectural Institute of Public Health and the Japan Weather Association reported that much JCP was scattered in the spring of 2005, and the level of JCP reached 10–30 pollen/cm² on the first 10 days of March (4–5 weeks after the start of ingestion), being maximal (approx. 600 pollen/cm²) in the last 10 days of March (7 weeks). Thereafter, the amount of scattered JCP gradually declined, although it was still detected at more than 10–30 pollen/cm² until the beginning of April (9 weeks). Furthermore, hinoki pollen started to scatter from the beginning of April, causing a biphasic change in the average amount of total pollen in the spring of 2005 (fig. 2).

Study Population

One hundred and twenty subjects were enrolled, but 11 subjects declined to take part in the study for personal reasons. As a result, 109 subjects (54 subjects in the placebo group and 55 in the LcS group) completed the study and their data were analyzed (fig. 1). There was no difference between the groups in terms of age, total and anti-JCP IgE level, and severity of allergic symptoms before the study (table 1).

Subjective Symptoms

Nasal and ocular SMS began to rise during the first 10 days of March in 2005 (4 weeks after the start of ingestion) and increased in association with the increase in scattered JCP. Deterioration of nasal SMS was delayed 1 week in the LcS group compared with the placebo group, but the difference in nasal SMS between the groups was not significant during the study (fig. 2). However, when the patients were divided into two categories ('mild' and

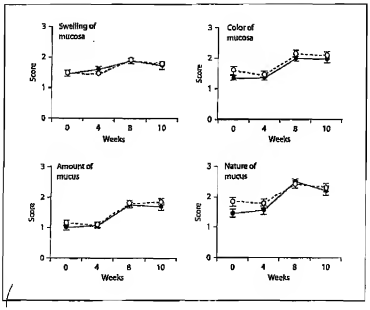

Fig. 3. Score of condition of nasal mucosa throughout the study. Data are presented as means ± SE. ● = LcS group; ○ = placebo group.

'moderate-to-severe') based on the nasal symptom score before the study, the nasal SMS in moderate-to-severe cases in the LcS group was lower at 4 and 5 weeks than in the placebo group (LcS group n = 13; placebo group n = 11; data not shown).

Medical Examination of Nasal Cavity

Scores of swelling and redness of the nasal mucosa, and the amount and nature of mucus deteriorated in association with the increase in scattered JCP. However, none of these scores differed between the groups (fig. 3).

Blood Examination

Immunological parameters associated with allergic symptoms increased in response to the amount of scattered JCP. Compared with the value before ingestion, the ECP level rose by 4 weeks and the anti-JCP IgE level and eosinophil number increased by 8 weeks. No difference was detected in immunological parameters between the two groups during the study (fig. 4). We divided the participants into mild cases and moderate-to-severe cases based on the nasal symptom score before the study and performed the statistical analysis, but there was no significant difference between the two groups (data not shown).

Side Effects

Ten subjects suffered from a cold, 3 subjects developed diarrhea, and 1 subject vomited during the study. All of these disorders were transient and there was no concern that they were related to the ingestion of fermented milk containing LcS (data not shown).

Discussion

We investigated the effect of fermented milk containing LcS on allergic symptoms triggered by JCP in a randomized double-blind, placebo-controlled study. The results showed that nasal and ocular SMS worsened in accordance with the increase in scattered JCP, confirming that the subjective symptoms of allergic rhinitis appear swiftly in response to scattered JCP. We have also performed a similar study in the spring of 2004 when a very small amount of JCP was scattered. Most patients did not experience any problem related to allergic symptoms, and nasal and ocular SMS in the participants did not rise in the spring of 2004 (data not shown). Taken together, these observations demonstrate that subjective symptoms are good parameters to assess allergic rhinitis during the JCP season.

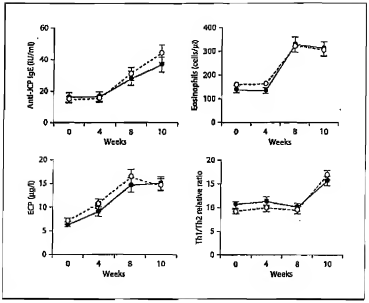

Fig. 4. Immunological parameters throughout the study period. Data are presented as means ± SE. ● = LcS group; ○ = placebo group.

Nasal and ocular SMS were not different between the LcS group and the placebo group. These results show that supplementation with fermented milk containing LcS during the JCP season does not prevent the exacerbation of seasonal allergic rhinitis. When the subjects were divided into two subgroups based on the severity of nasal symptom score before the start of ingestion, nasal SMS of the participants with moderate-to-severe nasal symptom scores was lower in the LcS group than in the placebo group at 4 weeks and 5 weeks, indicating that LcS may delay the onset of allergic symptoms in patients with a moderate-to-severe condition. In previous studies, we found that a daily drink of 80 ml of fermented milk containing LcS caused recovery of natural killer cell activity in humans [17–19]. Therefore, we considered that 80 ml of fermented milk containing LcS could be sufficient for modulating human immune function. However, it remains to be elucidated whether more than 80 ml may improve the results in placebo-controlled trials using participants with moderate-to-severe nasal symptoms in future investigations.

The clinical effects of probiotics on allergic rhinitis have been examined in several studies. Fermented milk containing *L. paracasei* LP-33 reduced the subjective symptoms in patients with perennial allergic rhinitis more efficiently than fermented milk containing *Streptococcus thermophilus* and *L. bulgaricus* [6]. Fermented milk containing *L. acidophilus* L-92 improved the subjective symptoms in not only perennial allergic rhinitis, but also Japanese cedar pollinosis, compared with heat-treated milk, although the immunological parameters were not different between the L-92 group and the placebo group [7, 8]. Furthermore, the clinical symptoms in Japanese cedar pollinosis were relieved in patients ingesting yogurt fermented with *B. longum* BB536, *S. thermophilus*, and *L. delbrueckii* compared with those ingesting yogurt fermented with *S. thermophilus* and *L. delbrueckii* and the immunological parameters were modulated by *B. longum*-BB536-fermented yogurt [9]. These results suggest that some probiotic strains and/or their fermentation products are responsible for improvement of allergic rhinitis. In contrast, supplementation with capsules containing *L. rhamnosus* GG did not improve the clinical symptoms of birch-pollen-allergic patients. Therefore, further investigation is required to elucidate the antiallergic effects of probiotics on allergic rhinitis. Wilhan et al. [21] have reported that administration of *L. rhamnosus* GG did not improve the clinical severity in infants with food allergy, but this probiotic was considered effective for the improvement of symptoms in healthy term

tized subgroup. Our results also suggest that supplementation with LcS might delay the onset of allergic symptoms in subjects with moderate-to-severe symptom scores. Altogether, the effect of probiotics on allergic rhinitis may vary dependent on the disease condition.

The effects of probiotics to modulate immunological parameters associated with allergic symptoms should be elucidated. It was reported that LcS suppressed IgE production in vitro and reduced the antiovalbumin IgE level in wild-type mice and transgenic mice expressing ovalbumin-specific T-cell receptor [10–12]. However, no difference was detected in immunological parameters between the LcS group and the placebo group in this clinical trial. Probiotics may possibly improve subjective symptoms even if immunological parameters such as the allergen-specific IgE level or Th1/Th2 imbalance are not normalized. In that sense, it is of great interest that supplementation with *L. rhamnosus* 19070–2 and *L. reuteri* DSM stabilized the intestinal barrier function and decreased gastrointestinal symptoms in children with atopic dermatitis [22]. Moreover, involvement of natural killer T cells and regulatory T cells in the induction and control of allergic responses has been proposed [23, 24]. Therefore, other mechanisms besides suppression of IgE production or normalization of Th1/Th2 imbalance could be involved in the antiallergic activity exerted by probiotics in humans.

Acknowledgements

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ATTACHMENT D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Naoyuki YAMAMOTO, et al.

Serial No. 10/518,801 Examiner: Vera Afremova

Filed: January 26, 2005 Group Art Unit: 1657

For:

RULE 132 DECLARATION

I, Shigeru FUJIWARA, a Japanese citizen having an address at c/o Functional Food & Drink Development Laboratory, Calpis Co., Ltd., 5-11-10, Fuchinobe, Sagamihara-shi, Kanagawa, Japan, declares as follows:

1. I am a researcher working with the inventors of the above-identified patent application since September 2002.

2. In March 1982, I graduated from University of Yamanashi, where I was enrolled at Faculty of Engineering, Department of Fermentative Production. In April 1982, I was employed by Snow Brand Milk Products Co., Ltd., where I was enrolled at Technical Research Institute. I had worked for Snow Brand Milk Products Co., Ltd. until August 2002 as a researcher, group leader, and line manager, in where my main work is researches about intestinal colonization and physiology of lactic acid bacteria. During this period, from 1985 to 1986, I was also a research student at RIKEN, where I was enrolled at Laboratory of Animal Pharmacology. In 1987, I was an International Frontier Research System researcher at RIKEN. From 1991 to 1993, I had been attached to Research Institute of Hospital for Sick Children in Toronto, Canada, in where I was enrolled at Department of Biochemistry, Dr. Janet F. Forstner's Laboratory. In 2000, I received a Ph.D degree from Faculty of Engineering, University of Yamanashi. In May, 2002, I received Research Award from the Japan Bifidus Foundation. In September, 2002, I was employed by Calpis Co., Ltd., in where I was enrolled at Food Research Laboratory as a manager until March 2005. Since April 2005, I have been involved in researches at Functional Food & Drink Development Laboratory of the same company as a senior manager, where my main work is researches about anti-allergic effect of lactic acid bacteria CL92 strain and

functions of lactic acid bacteria on health. Since 2006, I have also been a part-time lecturer at Aoyama Gakuin University.

3. I am a co-author of "Effect of Milk Fermented with *Lactobacillus acidophilus* Strain L-92 on Symptoms of Japanese Cedar Pollen Allergy: A Randomized Placebo-Controlled Trial", Biosci. Biotechnol. Biochem. 69(9), 2005, p1652-1660; "Clinical Effects of *Lactobacillus acidophilus* Strain L-92 on Perennial Allergic Rhinitis: A Double-Blind, Placebo-Controlled Study", J. Dairy Sci. 88, 2005, p527-533; "*Lactobacillus acidophilus* Strain L-92 Regulates the Production of Th1 Cytokine as well as Th2 Cytokines", Allergology International 56(3), 2007, p293-301; and many others, and an author of "*Bifidobacterium longum* SBT2928 and its Biological Significance", Bioscience and Microflora 21(4), 2002, p225-238, and many others.

4. I am familiar with the specification and the claims of this application.

5. I have conducted the following experiment under my direct supervision.

Experiment

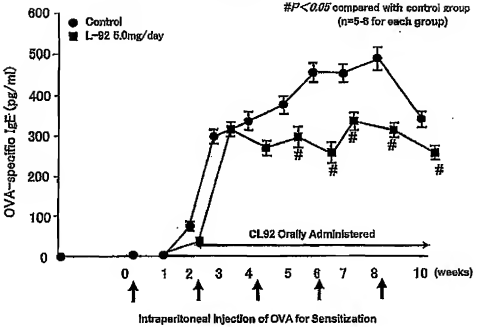
5.0 mg (7.5×10^9 cells) of lactic acid bacteria of the strain *Lactobacillus acidophilus* CL92 were suspended in water. The suspension was sterilized under heating at 100 °C for 10 minutes to prepare a suspension of killed CL92.

10 μg of ovalbumin (OVA) and 2 mg of aluminum hydroxide as an adjuvant were suspended in 300 μl of saline.

Two groups of BALB/c male mice, 5 to 6 animals per each group, were sensitized (immunized) with the OVA suspension every two weeks. From the day of the second sensitization, mice of one group were orally administered daily with the suspension of killed CL92. Mice of the other group were orally administered daily with only water instead of the suspension, as a control group.

Blood samples were obtained from the ophthalmic veins of the mice every week from the start of the sensitization, and serum samples were obtained. The OVA-specific IgE level in the serum samples was measured by means of sandwich ELISA in the same way as described under the title "Measurement of Blood OVA-IgE" in Example 1 in the present specification. The results are shown in Fig. 1.

Fig. 1: Change of Serum OVA-Specific IgE of OVA-Sensitized Mice and Effect of Oral Administration of CL92 Killed Cells



6. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United State Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Declared and signed at Kanagawa, Japan
Dated on 14th of February , 2008

Shigeru FUJIWARA

ATTACHMENT E

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Lactobacillus acidophilus strain L-92 induces apoptosis of antigen-stimulated T cells by modulating dendritic cell function

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Abstract

Beneficial effects of lactobacilli have been reported for patients with allergic diseases and intestinal disorders such as inflammatory bowel disease. However, it is not fully understood how such bacteria influence the immunologic response. For this purpose, we investigated the effect of *Lactobacillus acidophilus* strain L-92 (L-92) on antigen-stimulated T cell responses *in vitro* and *in vivo*. *In vitro*, L-92 decreased the proliferation of $CD4^+$ T cells stimulated with antigen, and also induced apoptosis of antigen-stimulated T cells. On the other hand, interferon (IFN)-γ secretion from naïve T cells was increased while interleukin (IL)-4 secretion was decreased by L-92. Co-culture with L-92 induced apoptosis of differentiated Th1 and Th2 cells. The degree of apoptosis induction was higher in Th2 cells. Moreover, L-92 up-regulated the expression of B7-H1 and down-regulated that of B7-H2 on dendritic cells (DCs), and DCs exposed to L-92 also induced apoptosis of antigen-stimulated T cells. Finally, orally administered L-92 induced apoptosis of OVA-specific TCR Tg T cells. These results indicate that L-92 attenuates the $CD4^+$ T cell response by inducing DC-mediated apoptosis and that it might exert beneficial effects in patients with diseases resulting from a hyper-response of $CD4^+$ T cells.

Keywords: *Lactobacillus acidophilus*; Apoptosis; T cells; Dendritic cells

Introduction

Lactic acid bacteria (LAB) have attracted a lot of interest because of their beneficial effects, such as their protective effect against intestinal infection (Servin 2004; Perdigon et al. 1999), their contribution to the treatment of inflammatory bowel diseases (IBD) (Setoyama et al. 2003), and the enhancement of natural killer cell activity (Gill et al. 2001). Moreover, LAB alleviate allergic diseases such as atopic dermatitis (Kalliomaki et al. 2001), perennial allergic rhinitis (Ishida et al. 2005a), and pollen allergy (Ishida et al. 2005b). Thus, some probiotic bacteria are capable of improving immunologic function.

Naïve T cells, which are presented antigen by antigen-presenting cells (APC), produce IL-2, proliferate, and

Abbreviations: AICD, activation-induced cell death; APC, antigen-presenting cells; DCs, dendritic cells; IFN, interferon; IL, interleukin; LAB, lactic acid bacteria; TLR, Toll-like receptors.

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develop into effector T cells. These activated T cells undergo apoptosis, which is known as activation-induced cell death (AICD) and plays an important role in maintaining T cell homeostasis (Russell et al. 1991; Ju et al. 1995). After the initial activation, naïve T cells differentiate into Th1 or Th2 cells and these secrete different cytokines (Abbas et al. 1996). Th1 cells secrete, among others, IFN-γ that principally mediates the cell-mediated immune response. Th1 responses can also trigger the pathogenesis of inflammatory and autoimmune disease such as type 1 diabetes and IBD (Liblau et al. 1995). Th2 cells secrete IL-4 and IL-5, among others, which mediate humoral immunity. Th2 type cytokines play a role in the differentiation into IgE antibody-producing B cells as well as in the recruitment of mast cells and eosinophils. As a result, Th2 cells will trigger the development of allergic diseases (Romagnani 1994). T cell activation and differentiation are usually a consequence of antigen presentation by dendritic cells (DCs) of major histocompatibility complex class II (MHC class II) molecules in combination with proper expression of costimulatory molecules and the secretion of specific cytokines (Banchereau and Steinman 1998).

LAB modulate the phenotype and functions of DCs (Mohamadzadeh et al. 2005). Lactobacilli up-regulate the expression of MHC class II and costimulatory molecules, and induce some cyokines including IL-12 (Drakes et al. 2004). IL-12 induced by lactobacilli skews naïve T cells toward Th1 (Shida et al. 1998; Pochard et al. 2005). As a result, lactobacilli inhibit antigen-induced IgE production (Shida et al. 1998; Ishida et al. 2003). These results suggest that LAB modulate T cell function by modulating DC function. Indeed, *Lactobacillus rahmnosus* modulates DC function to induce a form of T cell hyporesponsiveness (Braat et al. 2004).

In this study, we examined the effect of *Lactobacillus acidophilus* L-92 on antigen-stimulated $CD4^+$ T cell function. We showed that *L. acidophilus* induced apoptosis of antigen-stimulated T cells *in vitro* and *in vivo*. Our data suggest that *L. acidophilus* attenuates the T cell response, and that these phenomena might explain the beneficial effects of lactobacilli in patients with immune diseases.

Materials and methods

Mice

DO11.10 mice (8–14 weeks) (Murphy et al. 1990) transgenic for $OVA_{323-339}$-specific and $I\text{-}A^d$-restricted TCR-αβ with a BALB/c genetic background were used in these experiments. Female BALB/c mice (9–10 weeks) were purchased from Clea Japan Inc. (Tokyo, Japan).

Preparation of bacteria

Lactobacillus acidophilus strain L-92 was cultured at 37 °C for 18 h in Lactobacilli-MRS broth (DIFCO, Detroit, MI), washed with distilled water and then lyophilized. Lyophilized bacteria were suspended in PBS and killed by heating at 100 °C for 10 min.

Preparation of $CD4^+$ T cells, APC, and $CD11c^+$ DCs

DO11.10 OVA-specific TCR transgenic $CD4^+$ T cells were isolated from splenocytes by magnetic activating cell sorter (MACS, Miltenyi Biotec, Bergish Gladbach, Germany) positive selection using CD4 microbeads (Miltenyi Biotec). T cell-depleted splenocytes as APC were isolated from splenocytes of BALB/c mice by MACS negative selection using Thy1.2 microbeads (Miltenyi Biotec). DCs were purified from splenocytes of BALB/c mice by MACS positive selection using CD11c microbeads (Miltenyi Biotec).

Cell culture

Cells were cultured in RPMI1640 containing 5% fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 0.03% glutamine in the absence or presence of L-92 (0.1, 1, 10 μg/ml).

ELISA for cytokine

$CD4^+$ T cells (1×10^5 well^{-1}) were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA (SEIKAGAKU Corporation, Japan) in the presence (0.1, 1, 10 μg/ml) or absence of L-92 for 72 h. Cytokine levels in culture supernatants were determined by ELISA as previously described (Ise et al. 2000). Briefly, capture antibodies (BD PharMingen, San Diego, CA) were coated on ELISA plates. After washing and blocking the plates, samples and standards were added. After washing, biotinylated antibodies (BD PharMingen) were added. The wells were washed, and streptavidin-conjugated alkaline phosphatase (Zymed, South San Francisco, CA) was added. The wells were washed and incubated with disodium 4-nitrophenylphosphate solution. Optical densities were read at 405 nm.

In vitro proliferation assay

$CD4^+$ T cells (1×10^5 well^{-1}) were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence (0.1, 1, 10 μg/ml) or absence of L-92 for 72 h. Then cultures were pulsed with 1 μCi/well [^{3}H]-thymidine for 24 h and

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then harvested. Proliferation was assessed based on the incorporation of [^{3}H]-thymidine.

Annexin V staining

$CD4^+$ T cells (1×10^5 well^{-1}) were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence (0.1, 1, 10 μg/ml) or absence of L-92 for 96 h. Cells were harvested and washed with FACS buffer (1% FCS and 0.1% NaN_3 in PBS), incubated with anti-CD16/32 (2.4G2, BD PharMingen) on ice to block non-specific binding to Fc receptors, and then stained with KJ1. 26-FITC. Cells were further stained using Annexin V-PE Apoptosis Detection Kit I (BD PharMingen) and analyzed using a FACS LSR with CellQuest software (BD Biosciences, Mountain View, CA).

TUNEL assay

$CD4^+$ T cells (1×10^5 well^{-1}) were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence (10 μg/ml) or absence of L-92 for 96 h. The MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan) was used according to the manufacturer's suggestions. Briefly, cells were incubated with anti-CD16/CD32 to block non-specific binding to Fc receptors, and then stained with KJ1.26-PE (Caltag, California, USA). Cells were fixed with 4% paraformaldehyde in PBS, and then incubated in 70% EtOH at −70 °C to permeabilize. After washing, cells were incubated for 1 h at 37 °C with TdT reaction reagent (FITC-dUTP and TdT) and analyzed using a FACS LSR with CellQuest software.

Preparation of Th1 and Th2 cells

$CD4^+$ T cells from DO11.10 mice were cultured with 1 mg/ml OVA and APC treated with 50 μg/ml mitomycin C (Sigma). For Th1 polarization, anti-IL-4 (11B11, 5 μg/ml) and recombinant IL-12 (2 ng/ml) were added to the cultures and for Th2 polarization, anti-IL-12 (C17.8, 5 μg/ml) and recombinant IL-4 (2 ng/ml) were added. We used harvested cells after 7 days culture as Th1 cells and Th2 cells. Th1 cells and Th2 cells were cultured with APC and 1 mg/ml OVA in the absence or presence of 10 μg/ml L-92.

Flow cytometric analysis of DCs surface markers

DCs (5×10^5 cells/ml) from BALB/c mouse spleen were cultured in the absence or in the presence of 10 μg/ml of L-92 for 24 h. Cultured cells were harvested and incubated with anti-CD16/32 and stained with anti-CD11c-FITC (N418) and PE-labeled anti-B7-H1 (MIH5, *e*-Bioscience, San Diego, CA) or PE-labeled anti-B7-H2 (HK5.3, *e*-Bioscience). Dead cells were excluded by propidium iodide staining. Data were gated on viable $CD11c^+$ cells.

Apoptosis induction *in vivo*

DO11.10 mice were intraperitoneally injected 100 μg of OVA in complete Freund's adjuvant (CFA) on day 0. Then they were administered heat-treated L-92 by gavage at a dose of 10 mg/day, or saline, for 4 days. The mice were sacrificed and splenocytes were isolated. Then these were incubated with anti-CD16/32 on ice and stained with anti-CD4-APC (BD Pharmingen) and KJ1.26-FITC. Apoptosis of KJ1.26 positive cells was determined using the Annexin V-PE Apoptosis Detection Kit I.

Statistical analysis

Differences in the ratio of apoptotic cells or the cytokine levels between absence of L-92 were analyzed by using one way ANOVA followed by Dunnett's or Tukey's multiple comparison test. The Student's *t*-test was used to analyze the effect on differentiated T cells.

Results

L-92 inhibits the proliferative response of antigen-stimulated T cells and induces apoptosis of these cells *in vitro*

To study the effect of *L. acidophilus* L-92 on antigen-stimulated $CD4^+$ T cell function, we first investigated IL-2 secretion from these cells. $CD4^+$ T cells from DO11.10 mice were cultured with APC and OVA in the presence or absence of heat-treated L-92 for 72 h. IL-2 secretion from antigen-stimulated $CD4^+$ T cells was decreased by L-92 treatment (Fig. 1A). Moreover, we determined the proliferative activity of antigen-stimulated T cells by measuring [^{3}H]-thymidine incorporation. As shown in Fig. 1B, heat-treated L-92 inhibited [^{3}H]-thymidine incorporation in a dose-dependent manner. We considered the possibility that the inhibition of T cell proliferation by L-92 was due to the induction of apoptosis. Thus, we examined whether L-92 induced apoptosis of antigen-stimulated T cells.

A marker of apoptosis is the appearance of phosphatidylserine (PS) on the outer membrane, a process that is called membrane-flip. PS can be detected by staining with annexin V. Splenic $CD4^+$ T cells from DO11.10 mice were incubated with APC and OVA in the presence or absence of L-92. Annexin V-positive, KJ1.26-positive cells were detected by flow cytometry. In the presence of L-92 (10 μg/ml), the number of apoptotic KJ1.26 cells gradually increased compared to that found in the

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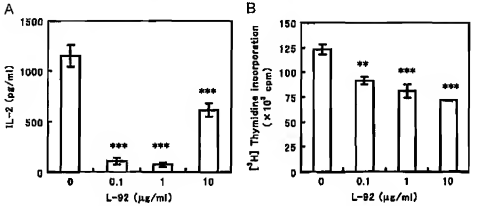

Fig. 1. L-92 inhibits the proliferative response of $CD4^+$ T cells. (A) $CD4^+$ T cells (1×10^5 well^{-1}) from DO11.10 OVA-specific TCR Tg mice were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence or absence of L-92 for 72 h. The levels of IL-2 was determined by ELISA. (B) $CD4^+$ T cells (1×10^5 well^{-1}) from DO11.10 OVA-specific TCR Tg mice were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence or absence of L-92 for 72 h. Then cultures were pulsed with [^{3}H]-thymidine for 24 h and [^{3}H]-thymidine incorporation was measured. Data are presented as the mean value ± S.E. $^{**}p<0.01$, $^{***}p<0.001$ vs. 0 μg/ml of L-92 (Dunnett's test). The results shown are representative of two independent experiments.

absence of L-92 in a 4-day culture period. Fig. 2A–C shows the ratio of apoptotic $CD4^+$ T cells at 96 h. Even at the dose of 1 μg/ml L-92 induced apoptosis of antigen-stimulated T cells but 10 μg/ml of *Escherichia coli* did not induce apoptosis (Fig. 2C). In addition to staining with annexin V, apoptosis was investigated by a TUNEL assay, which reveals the apoptotic fragmentation of nuclear DNA. The number of TUNEL-positive KJ1.26-positive cells was increased by L-92 treatment (Fig. 2D–F). These results demonstrate that L-92 induced apoptosis of antigen-stimulated T cells. The results imply that L-92 induced apoptosis of proliferating T cells activated by antigen stimulation, and that the inhibition of $CD4^+$ T cell proliferation was due to induction of apoptosis.

Effect of L-92 on IFN-γ and IL-4 secretion from naïve T cells

To investigate the effect of L-92 on cytokines secretion from naïve T cells, we measured the amount of IFN-γ and IL-4 secreted from $CD4^+$ T cells cultured with L-92. At low doses (0.1–1 μg/ml) L-92 enhanced IFN-γ secretion, but at 10 μg/ml L-92 did not (Fig. 3A). On the other hand, IL-4 secretion was decreased by L-92 treatment in a dose dependent manner (Fig. 3B).

L-92 induces apoptosis of differentiated Th1 and Th2 cells

Since we found that L-92 induced apoptosis of naïve T cells after antigen stimulation, we then investigated the effect of L-92 on differentiated Th1 and Th2 cells. Th1 and Th2 cells were prepared as described in Materials and Methods section. Apoptosis induction after antigenic stimulation in the presence or absence of L-92 was examined by annexin-V staining. L-92 induced apoptosis of Th2 cells after stimulation with antigen (Fig. 4A). Stimulation of Th1 cells with antigen in the presence of L-92 also resulted in an increased ratio of apoptotic cells (Fig. 4B), although the degree of apoptosis induction was lower compared to Th2 cells. These results suggest that L-92 induced apoptosis of differentiated helper T cells.

L-92 modulates the expression of costimulatory molecules on DCs and induces apoptosis of $CD4^+$ T cells

Costimulatory molecules expressed of DCs play an important role in T cell function, so we investigated whether L-92 modulate the expression of costimulatory molecules. B7-1 and B7-2 are well known costimulatory molecules. In preliminary experiments, we investigated the effect of their expression by L-92 treatment, but L-92 did not effect their expression (data not shown). Recently, new B7-family molecules have been identified, so we investigated the expression of other B7-family molecules, B7-H1 and B7-H2. DCs isolated from the spleen of BALB/c mice were treated with L-92 for 24 h, and the expression of costimulatory molecules was analyzed by flow cytometry. The expression of B7-H1 was up-regulated by L-92 treatment (Fig. 5A), while that of B7-H2 was down-regulated (Fig. 5B). It is known that B7-H1 negatively regulates T cell responses (Freeman et al. 2000), and B7-H2 positively regulates T cell

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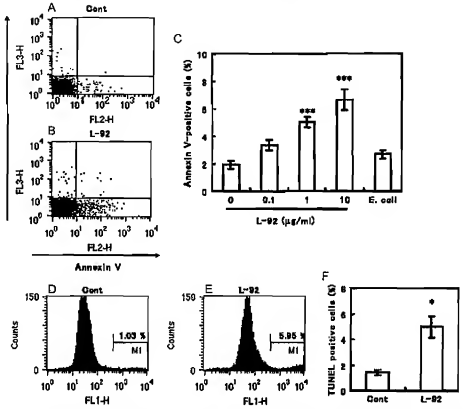

Fig. 2. L-92 induces apoptosis of antigen-stimulated $CD4^+$ T cells. $CD4^+$ T cells (1×10^5 well^{-1}) from DO11.10 mice were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the absence (A, D) or presence (B, E) of 10 μg/ml L-92 for 96 h. Cells were stained with KJ1.26-FITC, annexin V-PE, and 7-AAD (A, B and C) or KJ1.26-PE, and TdT + FITC-dUTP (D, E and F). The dot plots (A, B) and histograms (D, E) represent KJ1.26 positive cells. (C) The number of annexin V-positive, KJ1.26-positive cells increased in the presence of L-92 but did not in the presence of 10 μg/ml of *E. coli*. Data are presented as the mean value ± S.E. The results shown are representative of two independent experiments. $^{***}p < 0.001$ vs. 0 μg/ml of L-92 (Dunnett's test). (F) The number of TUNEL-positive, KJ1.26-positive cells increased in the presence of L-92. Data are presented as the mean value ± S.E. The results shown are representative of two independent experiments. $^{*}p < 0.001$ vs. control (Student's *t*-test).

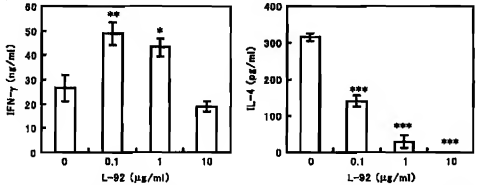

Fig. 3. Effect of L-92 on IFN-γ and IL-4 secretion from naïve T cells. $CD4^+$ T cells (1×10^5 well^{-1}) from DO11.10 mice were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the presence of L-92 for 72 h. Cytokines were determined by ELISA. Data are represented as the mean value ± S.E. The results shown are representative of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 0 μg/ml of L-92 (Dunnett's test).

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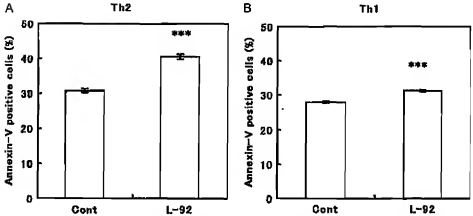

Fig. 4. L-92 induces apoptosis of differentiated Th1 and Th2 cells. Differentiated Th2 cells (A) and Th1 cells (B) (1×10^5 well^{-1}) were cultured with APC (3×10^5 well^{-1}) and 1 mg/ml OVA in the absence or presence of 10 μg/ml L-92 for 96 h. Annexin V-positive, KJ1.26-positive cells were determined by flow cytometric analysis. Data are presented as the mean value±S.E. $^{***}p<0.001$ vs. control (Student's *t*-test).

responses (Wang et al. 2000). These results suggest that L-92 might inhibit T cell function through a DC-mediated induction of apoptosis. Thus, we tested whether $CD4^+$ T cells co-cultured with DCs exposed to L-92 underwent apoptosis at a rate higher than normal. L-92 (10 μg/ml) induced apoptosis of $CD4^+$ T cells (Fig. 5C). The number of apoptotic cells in the presence of L-92 increased by increasing number of DCs (Fig. 5C). These results suggest that L-92 induced apoptosis of antigen-stimulated T cells through DCs.

L-92 induces apoptosis of antigen-stimulated $CD4^+$ T cells *in vivo*

We revealed that L-92 induced apoptosis of antigen-stimulated $CD4^+$ T cells *in vitro*. We thus investigated whether L-92 induces apoptosis of antigen-stimulated $CD4^+$ T cells *in vivo*. DO11.10 mice were intraperitoneally injected 100 μg OVA in CFA. Heat-treated L-92 (10 mg/day) were orally administered for 4 days. Then mice were sacrificed, and ratio of antigen-specific T cells and apoptotic cells were analyzed by flow cytometry. L-92 significantly increased apoptosis of antigen-specific (KJ1.26-positive cells) $CD4^+$ T cells in mice compared with the control group which received only intraperitoneal immunization with OVA in CFA (Fig. 6A). Antigen-specific $CD4^+$ T cells (KJ1.26-positive cells) were slightly decreased by administration of L-92 (Fig. 6B). There were no differences in the cytokine production (IFN-γ, IL-4 and IL-10) between the administration of L-92 and saline when splenocytes were restimulated with OVA (data not shown). These results show that L-92 induces apoptosis of antigen-stimulated T cells *in vivo*.

Discussion

Previous studies have shown that some *Lactobacillus* strains modulate DC function, and that LAB-exposed DCs inhibit the proliferation of allogenic T cells (Drakes et al. 2004; von der Weid et al. 2001) and CD3/CD28-stimulated T cell proliferation (Braat et al. 2004). In this study, we demonstrated that *L. acidophilus* L-92 inhibits the proliferation of antigen-stimulated $CD4^+$ T cells and induces apoptosis of activated T cells, as determined by annexin V staining and TUNEL assay. Our data suggest that L-92 induces apoptosis of T cells by modulating DC function. Finally, we showed that oral administration of L-92 induces apoptosis of antigen-stimulated T cells *in vivo*.

Engagement of the TCR/CD3 complex following response to an antigen results in T cell activation. The majority of activated effector T cells are eliminated by AICD. AICD occurs as a result of repeated stimulation through the TCR, up-regulating plasma membrane expression of Fas and its ligand (FasL), thereby acting as a feedback mechanism for terminating an ongoing immune response (Russell et al. 1991; Ju et al. 1995). Failure of T cells to undergo apoptosis may cause immune diseases. For example, T cells from Crohn's disease patients are resistant to IL-2 deprivation-induced apoptosis or Fas- and nitric oxide-mediated apoptosis (Ina et al. 1999). Moreover, apoptosis of lymphocytes from atopic patients cultured with a specific allergen does not increase, whereas lymphocytes from atopic patients receiving specific immunotherapy undergo apoptosis after culture with the allergen (Guerra et al. 2001). Therefore, apoptosis of T cells is important for the maintenance of T cell homeostasis.

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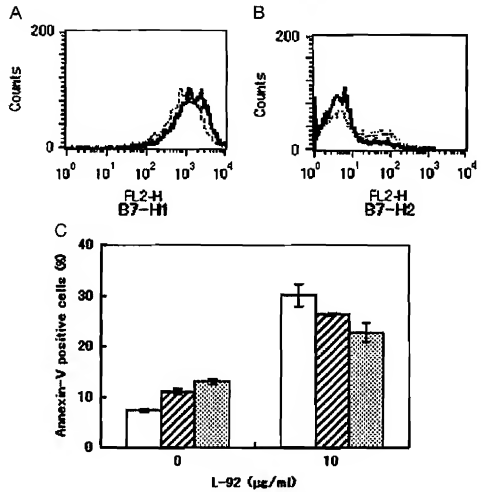

Fig. 5. L-92 modulates the expression of costimulatory molecules on DCs and DCs exposed to L-92 induce apoptosis of $CD4^+$ cells. DCs (5×10^5 cells/ml) from BALB/c mouse spleen were cultured in the absence (dotted lines) or in the presence of 10 μg/ml of L-92 (solid lines) for 24 h. Cells were stained with anti-CD11c-FITC and PE labeled anti-B7-H1 (A) or anti-B7-H2 (B). Data were gated on viable $CD11c^+$ cells. The results shown are representative of three independent experiments. (C) $CD4^+$ T cells (1×10^5 well^{-1}) from DO11.10 mice were co-cultured with DCs (opened bar: 5×10^5 well^{-1}, slash bar: 2×10^4 well^{-1}, dotted bar: 1×10^4 well^{-1}) and 1 mg/ml OVA for 96 h in the presence or absence of L-92 (10 μg/ml). Apoptotic KJ1.26-positive cells were determined by flow cytometric analysis. Data are presented as the mean value ± S.E.

We showed that *L. acidophilus* induced apoptosis of antigen-stimulated naïve $CD4^+$ T cells and differentiated helper T cells *in vitro* (Figs. 2 and 4) and *in vivo* (Fig. 6A). The decrease in $KJ1.26^+$, $CD4^+$ T cells (Fig. 6B) suggests that L-92 causes T cell deletion by inducing apoptosis of antigen-stimulated T cells. Lactobacilli are beneficial in patients with various inflammatory disorders and allergic diseases. And orally administered *L. acidophilus* L-92 has been shown to alleviate the symptoms of perennial allergic rhinitis (Ishida et al. 2005a), and pollen allergy (Ishida et al. 2005b). Our findings might explain the beneficial effect of LAB in patients affected by such diseases.

Costimulatory molecules expressed on DCs play important roles in T cell activation and tolerance. B7-1 (CD80) and B7-2 (CD86) interact with CD28 or CTLA-4; signaling through the B7/CD28 pathway is required for IL-2 production and T cell expansion, and signaling through the B7/CTLA-4 pathway delivers a negative signal (Wang and Chen 2004). Some probiotic bacteria up-regulate the expression of B7-1 and B7-2 (Mohamadzadeh et al. 2005; Drakes et al. 2004). Nevertheless, no difference in B7-1 or B7-2 expression was observed on splenic DCs after co-culture with L-92 (data not shown). Recently, several new members of the B7-family have been identified (Wang and Chen 2004).

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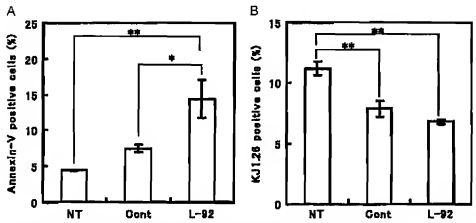

Fig. 6. L-92 induces apoptosis of antigen-stimulated CD4$^+$ T cells *in vivo*. DO11.10 mice were intraperitoneally injected with 100 μg of OVA in CFA on day 0. Then they were administered with heat-treated L-92 by gavage at a dose of 10 mg/day (L-92) or saline (control). After 4 days, mice were sacrificed. Spleen cells were stained with KJ1.26-FITC, anti-CD4-APC, annexin V-PE and 7-AAD. Annexin V-positive, CD4-, KJ1.26-positive cells were determined by flow cytometry (A). Percentage of CD4-, KJ1.26-positive cells as determined by flow cytometry (B). Data are presented as the means ± S.E. ($n = 3$). NT: not treated. $^*p<0.05$, $^{**}p<0.01$ (Tukey's HSD test). The results shown are representative of two independent experiments.

B7-H1 binds to its receptor: programmed death-1 (PD-1), and costimulation with B7-H1 inhibits TCR-mediated proliferation and cytokines production resulting in cell cycle arrest (Freeman et al. 2000). Moreover, B7-H1 on activated hepatic stellate cells enhances apoptosis of T cells (Yu et al. 2004). L-92 up-regulated B7-H1 expression on DCs (Fig. 5A). L-92 might induce apoptosis of T cells through B7-H1 present on DCs. Exposure to L-92 resulted in decreased expression of B7-H2, another B7 family molecule, which is a ligand for the inducible costimulator (ICOS) molecule. B7-H2 costimulates the proliferation and cytokine production of T cells (Wang and Chen 2004). Our results suggest that L-92 may inhibit the T cell response by modulating the expression of costimulatory molecules on DCs.

Commensal bacteria or orally administered bacteria interact with intestinal DCs (Macpherson et al. 2005). Intestinal DCs play an important role in innate and adaptive immune system (Niess and Reinecker 2005). It is well known that DCs influence the development of Th1 and Th2 responses, either by the cytokines they produce or by costimulating T cells, which can then proliferate and secrete cytokines and chemokines (Banchereau and Steinman 1998; Mellman and Steinman 2001). A recent study revealed that lactobacilli induce activation and maturation of DCs, and that lactobacilli-exposed DCs secrete proinflammatory cytokines such as IL-6, IL-8, IL-12, and TNF-α, as well as suppressive cytokines such as IL-10 and TGF-β (Mohamadzadeh et al. 2005; Drakes et al. 2004). L-92 also induced IL-6, IL-10, IL-12 from BALB/c splenocytes (data not shown), suggesting the possibility that L-92 may induce the secretion of these cytokines from DCs. IL-12 secreted by DCs skews naïve T cells toward Th1 polarization (Shida et al. 1998). In our experiment, L-92 induced Th1 cytokine secretion from naïve T cells albeit at low doses and suppressed Th2 cytokine secretion (Fig. 3). The up-regulation of the Th1 response by L-92 was reduced by neutralizing IL-12 (data not shown). Thus, two pathways may be involved in the inhibition of the Th2 response: induction of apoptosis, and differentiation to Th1. The effect on Th1 responses may be more complex. We consider that low dose of L-92 strongly enhanced IFN-γ secretion through induction of IL-12, while high dose of L-92 suppressed IFN-γ secretion by extensive induction of apoptosis. It has been shown that probiotic bacteria are beneficial for patients with IBD (Setoyama et al. 2003), which has been associated with elevated levels of Th1 cytokines in the colon (Powrie et al. 1994). In such cases, probiotic bacteria may suppress pathogenic Th1 conditions by enhancing the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, which may not only be secreted by DCs but also by regulatory T cells, or by inducing apoptosis as shown in this study. Our findings may also have relevance for the use of transgenic LAB as vehicles for therapy, such as the previous report utilizing IL-10-expressing bacteria (Steidler et al. 2000). It is possible that a combination of secretion of immunosuppressive cytokines and apoptosis induction may be effective.

Further studies are needed to elucidate active bacterial cellular components. We showed that *L. acidophilus* L-92 (Gram-positive bacteria) induced apoptosis of antigen-stimulated T cells but *E. coli* (Gram-negative bacteria) did not (Fig. 2C). Gram-positive bacteria and

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Gram-negative bacteria differ in their cell wall components. DC recognition of and response to molecular structures in bacteria is mediated by a family of pattern recognition receptors designated Toll-like receptors (TLRs). TLR signaling triggers a maturation program that includes up-regulation of MHC and costimulatory molecules and expression of several cytokines (Medzhitov 2001). Moreover, recent studies have suggested that TLRs may control T cell differentiation, activation and tolerance by modulating DC function (Barton and Medzhitov 2002; Kubo et al. 2004). CpG oligodeoxynucleotides (TLR9 ligand) administered together with antigen prevents Th2 cytokines induction, IgE production, and allergen-induced airway inflammation (Kline et al. 1998). It is likely that cellular component(s) of L-92 exert their T cell-inhibitory effects through TLRs.

In conclusion, *L. acidophilus* L-92 induced apoptosis of activated $CD4^+$ T cells and attenuated the $CD4^+$ T cell response. These phenomena could explain the beneficial effect of probiotic treatment in immunological diseases.

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